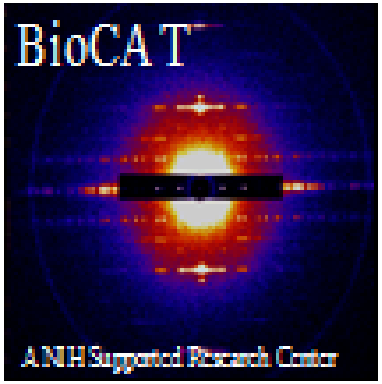


Sample Preparation Tutorial

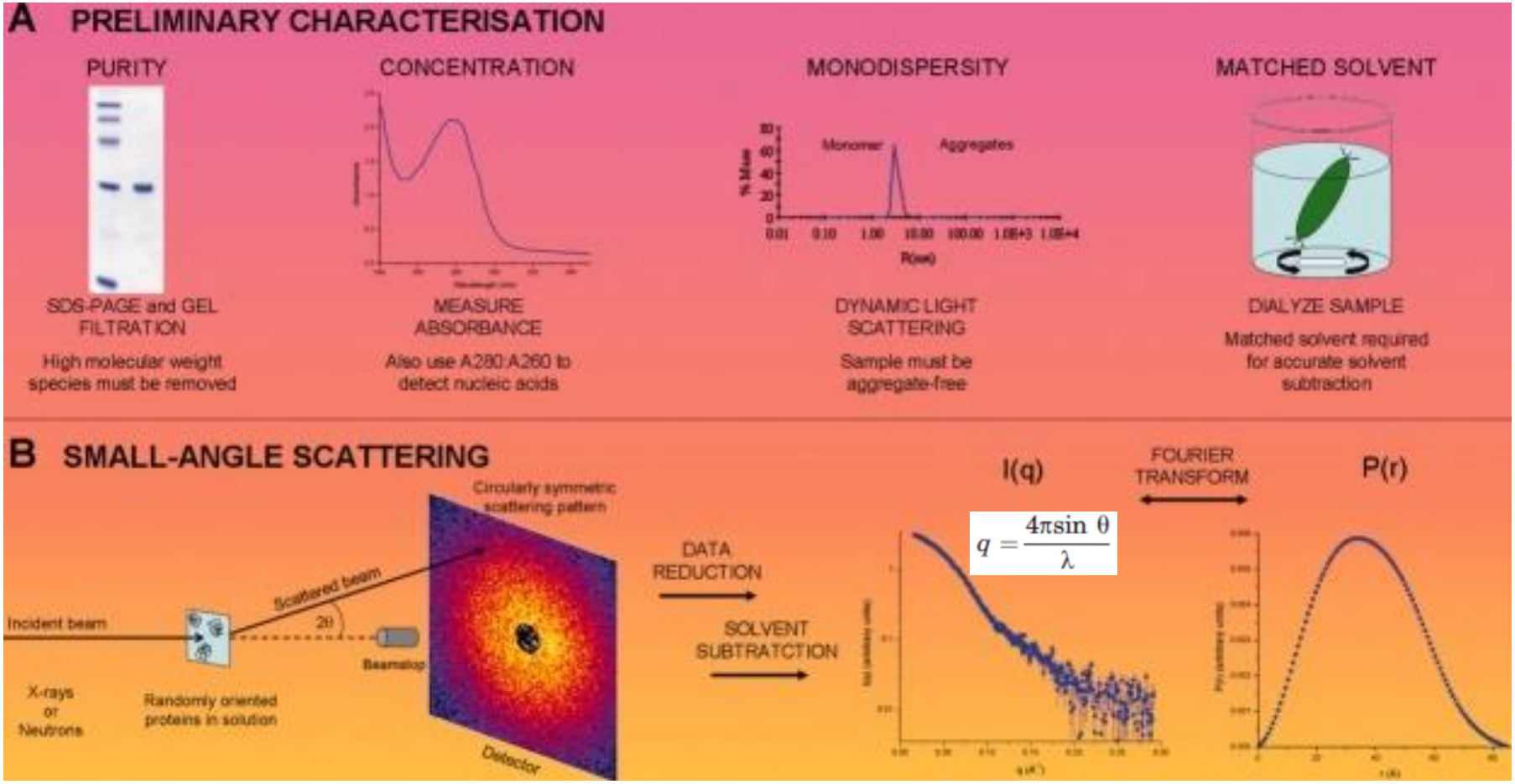
Srinivas Chakravarthy

BioCAT / Sector 18ID, APS

Illinois Institute of Technology

CSRRI
Center for Synchrotron Radiation Research & Instrumentation

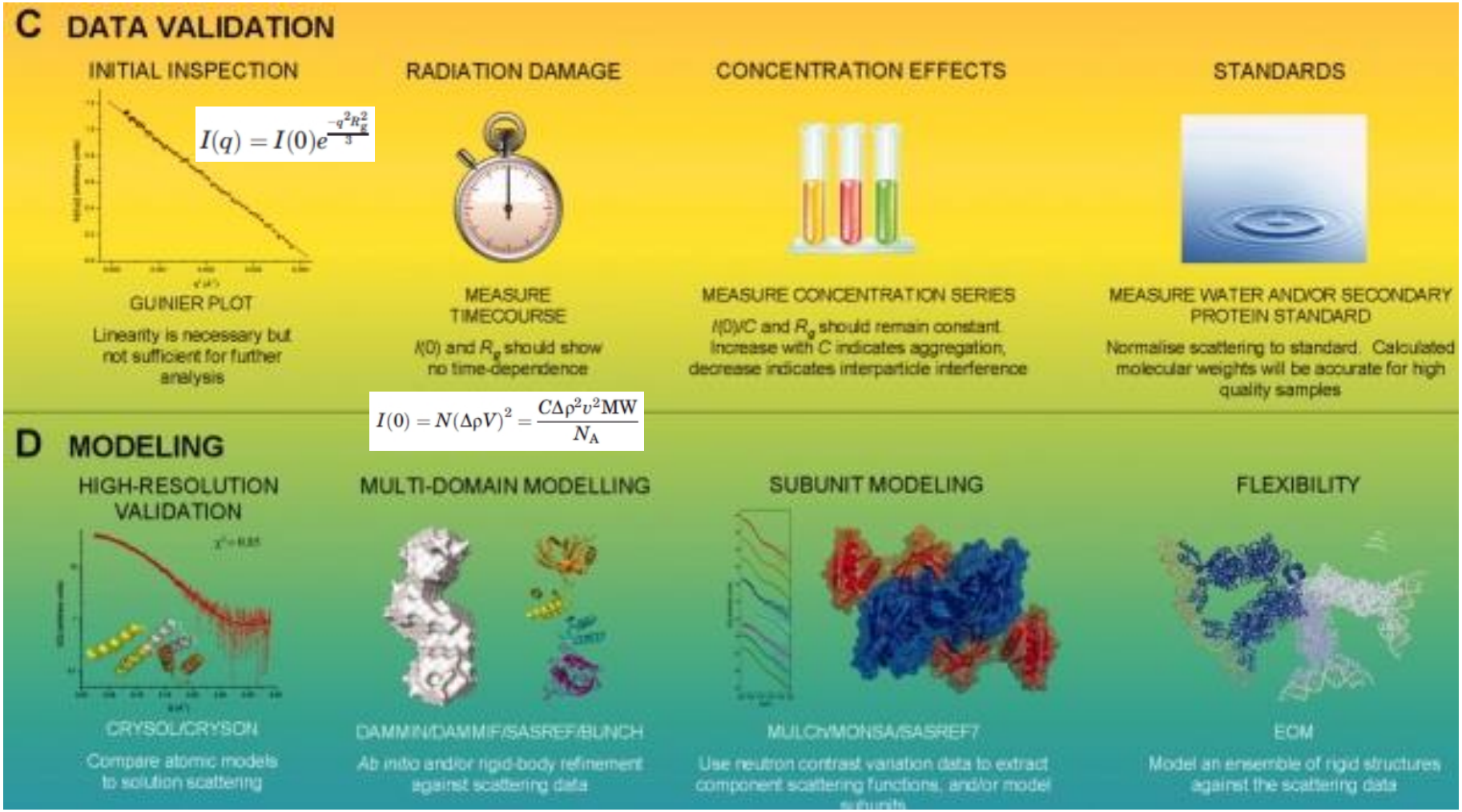
SAXS – The Basic Idea



$$R_g^2 = \frac{\int P(r) r^2 \mathrm{d}r}{2 \int P(r) \mathrm{d}r}, \qquad I(0) = 4\pi \int_0^{D_{max}} P(r) \mathrm{d}r.$$

Jacques and Trewhalla; Protein Science (2010) 19(4): 642–657

SAXS – The Basic Idea



Jacques and Trewhalla; Protein Science (2010) 19(4): 642–657

Most Encountered Sample Quality Issues

- Concentration effects (interparticle interaction or repulsion)
- Radiation Damage (Aggregation or Degradation)
- Incorrect Buffer Subtraction
- Polydisperse Sample

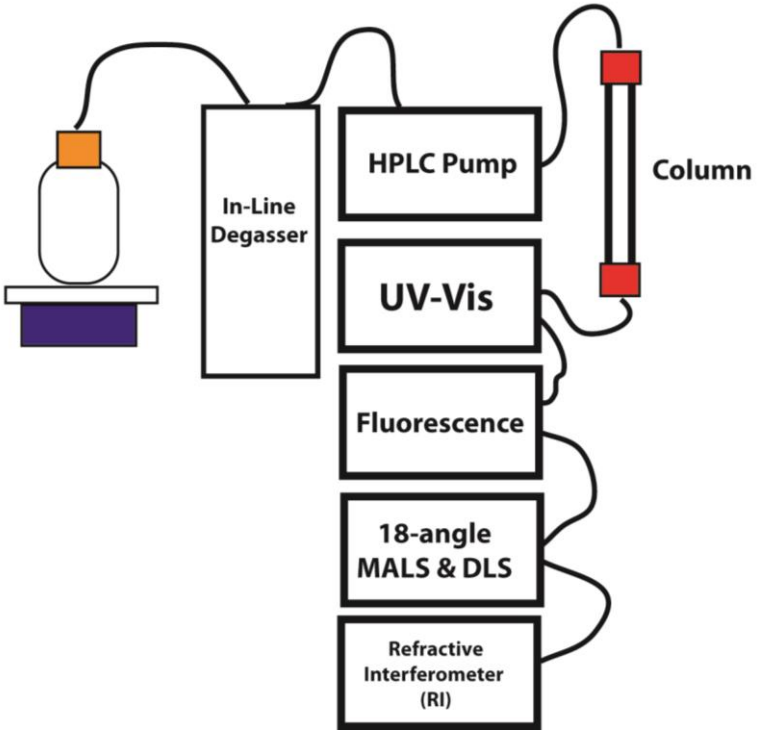
Preparation, Preparation, Preparation

- Arguably the most important part of a SAXS experiment is sample preparation.
- Optimization of the purification protocol.
- Biochemical / Biophysical characterization.
- Informed decisions towards the most suitable constructs (truncations / fusions).
- Talk extensively with the beam line staff before the trip about the experiment - eliminate guess-work from the experiment setup.

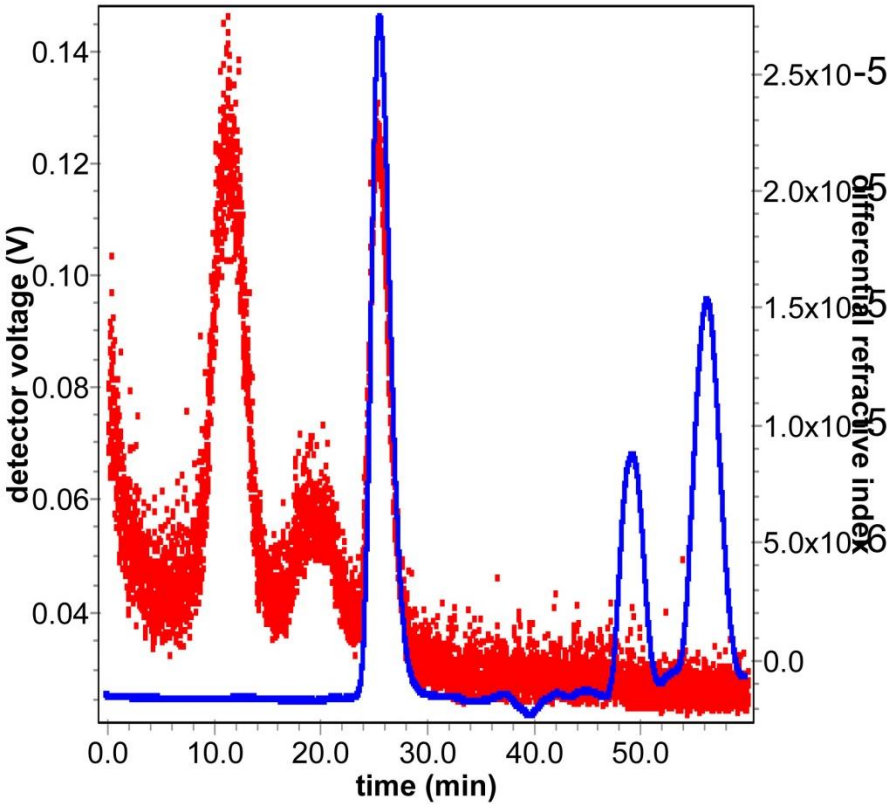
Diagnostics

- SDS-PAGE gels and native gels – Be wary though, no obvious contaminants does not always translate to pristine SAXS data.
- Symmetric single peaks obtained by size exclusion chromatography – But there may be shelf life issues and storage and shipping related complications.
- No visible particulates and precipitate and no significant loss of sample during concentration.
- DLS – often not very reliable.
- SEC-MALS and ultracentrifugation.

Size-Exclusion Chromatography In-Line with Multiple Angle Light Scattering

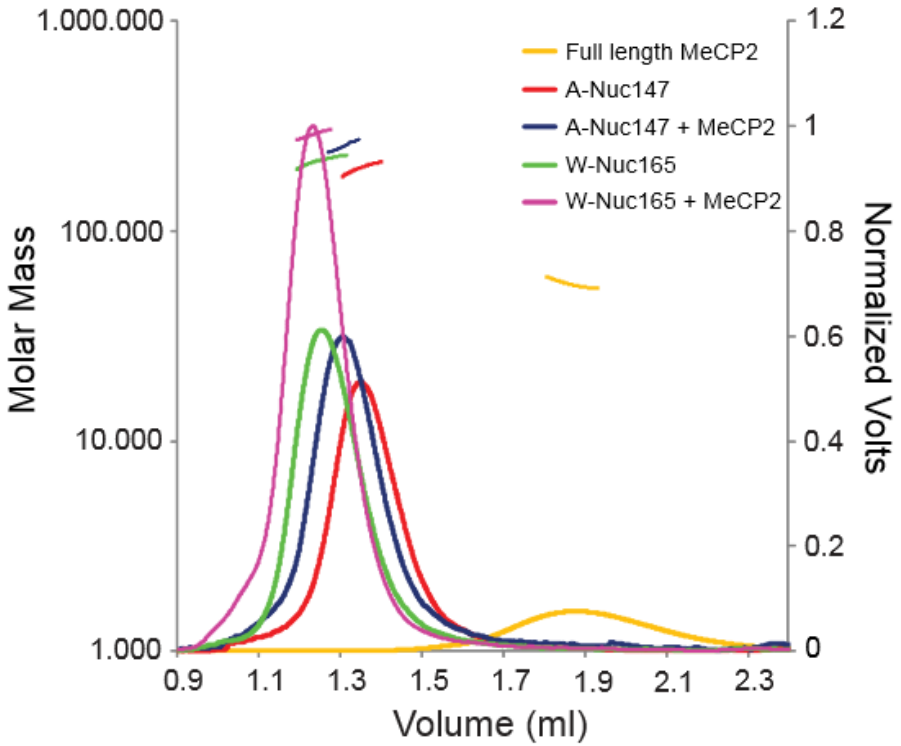


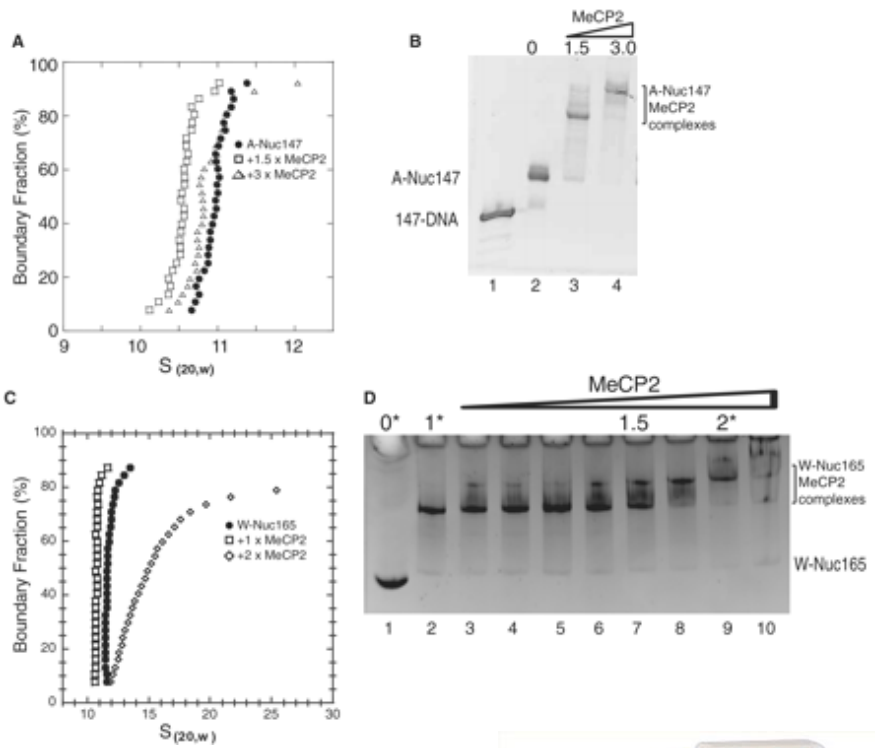
SEC-MALS Instrumentation Schematic Courtesy: Kushol Gupta (Univ. of Pennsylvania)



SEC-MALS with a nucleosome core particle Here demonstrates the need for assessing the quality of sample using a variety of techniques.

SEC-MALS and Analytical Ultracentrifugation as Complementary Techniques

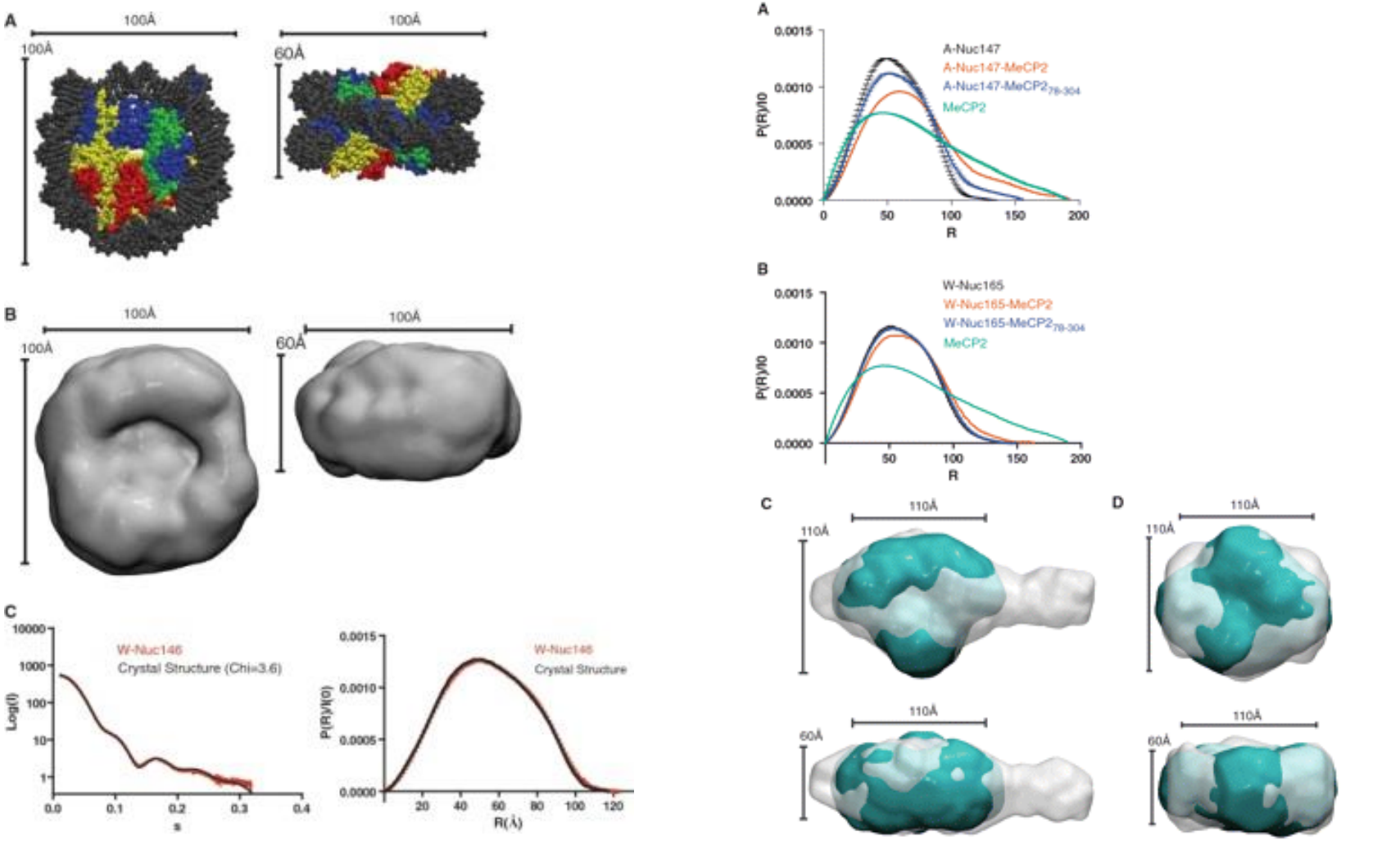




Molecule or complex	SEC-MALS Molecular weight (kDa)	
	Observed (%)	Calculated
BSA	66.47 ± 2	67.0
MeCP2 full length	56.00 ± 4	52.44
A-Nuc147	198.0 ± 2	205.0
A-Nuc147-MeCP2 full length	251.4 ± 2	257.9 (1:1)
W-Nuc165	202.8 ± 1	208.95
W-Nuc165-MeCP2 full length	253.9 ± 0.6	261.4 (1:1)

Yang, C et al., Nucleic Acids Res. 2011 May;39(10):4122-35. doi: 10.1093/nar/gkr005

Atomic Coordinates Compared to SAXS Data



Yang, C et al., Nucleic Acids Res. 2011 May;39(10):4122-35. doi: 10.1093/nar/gkr005

Optimal Sample Concentration

$$I(q) \propto \frac{N}{V} V_{particle}^2 (\rho_1 - \rho_2)^2 FF(q) S(q)$$

"solution part"

"protein part"

- Scattering intensity proportional to the molecular mass and the concentration.
- Dilution series, extrapolation to zero concentration, important to exclude concentration effects.
- Useful rule of thumb to estimate optimal concentration to start with – Molecular mass (Kda) * Concentration (mg/ml) ~ 100
- Generally 0.25-10 mg/ml (higher for smaller proteins and higher q-ranges, most beamlines use 30 – 100 ul sample volume.
- For LC-SAXS using a superdex-200 /75 (or a superose-3/6/12) 10/300 column (column volume = 24ml), we usually use ~ 200ul of 3-10mg/ml sample).
- Nucleic acids scatter more than proteins, so much better signal at lower concentrations.

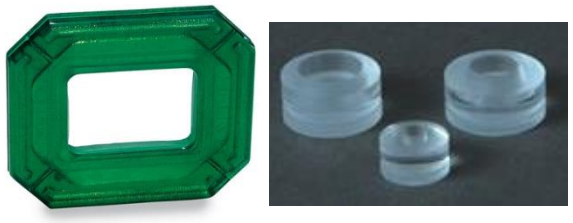
Sample Concentration Calculation

- Theoretical Extinction Coefficients can have upto 5-10% discrepancy.
- Experimental determination using denatured material (6M Guanidium HCl) - see ***Calculation of protein extinction coefficients from amino acid sequence data*** *Stanley C. Gill, a and Peter H. von Hippel Analytical Biochemistry Volume 182, Issue 2, 1 November 1989, Pages 319-326.*
- Bradford, Lowry, Biuret, and other colorimetric assays (use multiple concentrations and work with concentration-range relevant to the SAXS experiment).
- DNAseI digestion of polynucleotides.
- SAXS (I0) and SEC-MALS (RI).

Considerations for Buffer Composition

- Bring abundant amounts of MATCHED buffer (10X stocks when possible recommended for convenience of shipping and the opportunity to trouble shoot on site.
- Variety of buffer systems are suitable (Tris, HEPES, PBS etc…)
- Moderate salt concentrations – more than 1M NaCl not recommended but in extreme cases, can be done.
- Glycerol helps with prevention of radiation damage but higher concentrations than ~ 5% make buffer subtraction difficult and also decrease contrast.
- Avoid detergents. Try using concentrations well below CMC if you absolutely need them.
- Free radical scavengers such as DTT (1mM) or other reducing agents (BME, TCEP etc…), highly recommended (use fresh stocks).
- Retrieve used sample if possible for analysis in order to make better informed decisions during subsequent experiments.

Measures for Optimal Buffer Matching



Dialysis

SEC Buffer

Buffer Exchange

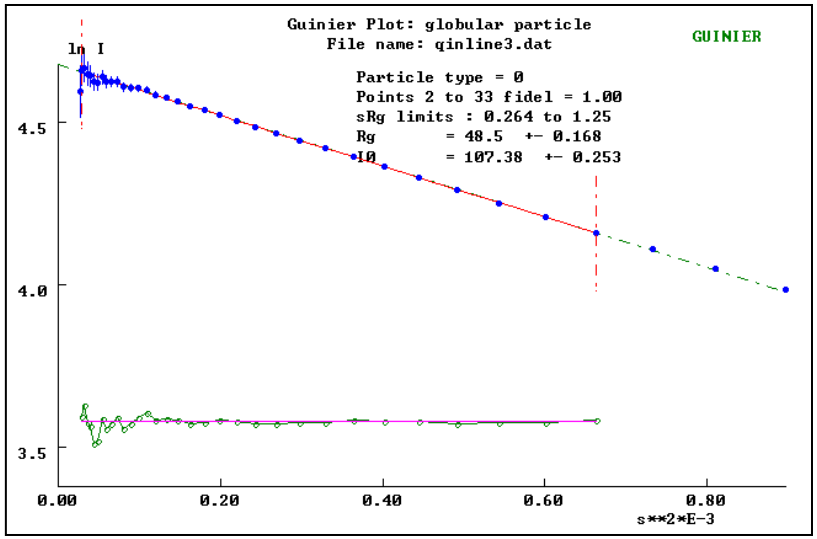
Inter-particle Interactions and Preventive Measures

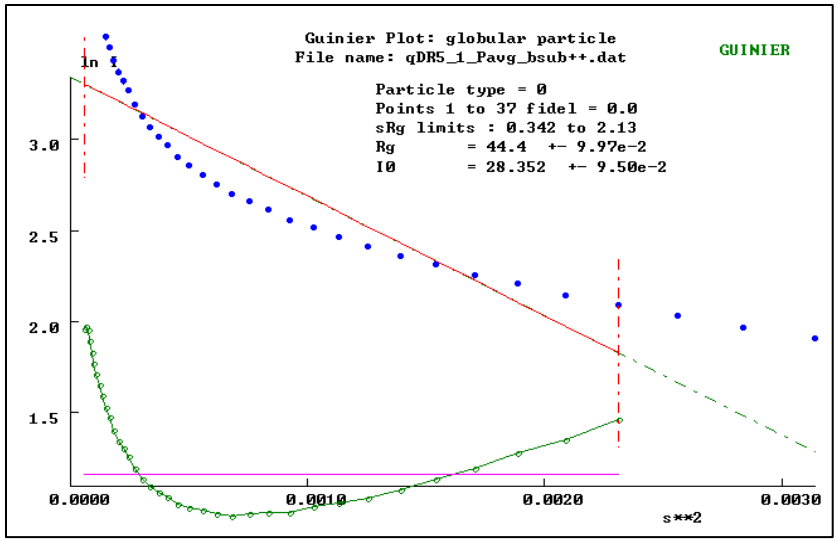
- Solvent exposed charged residues – electrostatic repulsion or attraction – can be neutralized by increasing salt in buffer.

- Hydrophobic interactions – can be ameliorated by moderate amounts (sub-CMC) of detergents.

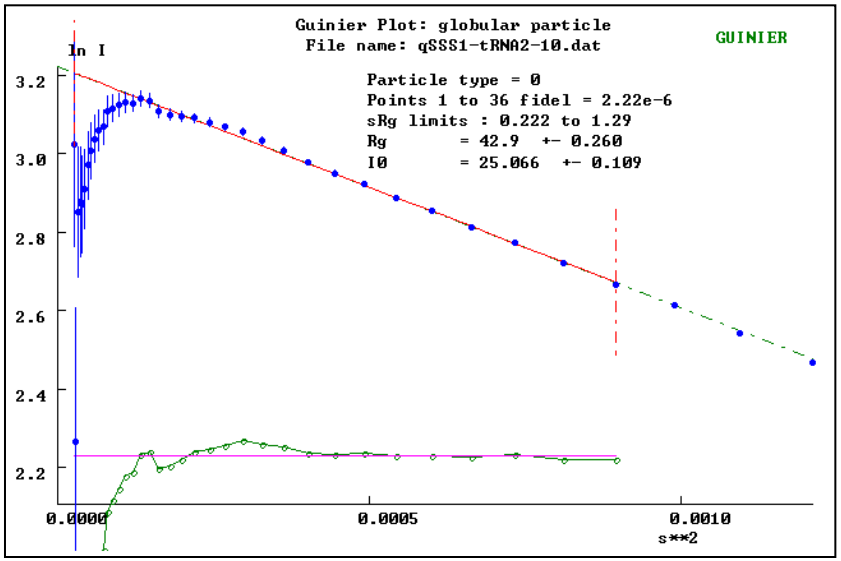
- Disulphide bonds – can be reduced by reducing agents such as DTT and TCEP.

Manifestation of Inter-particle Interactions in SAXS





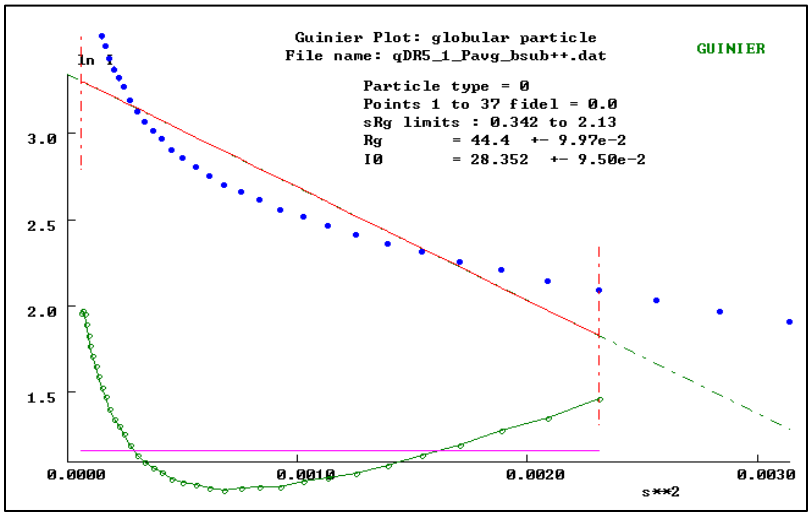
No Inter-Particle Interference



Aggregated

Inter-Particle Repulsion

Post-Aggregation First-Aid



Centrifugation

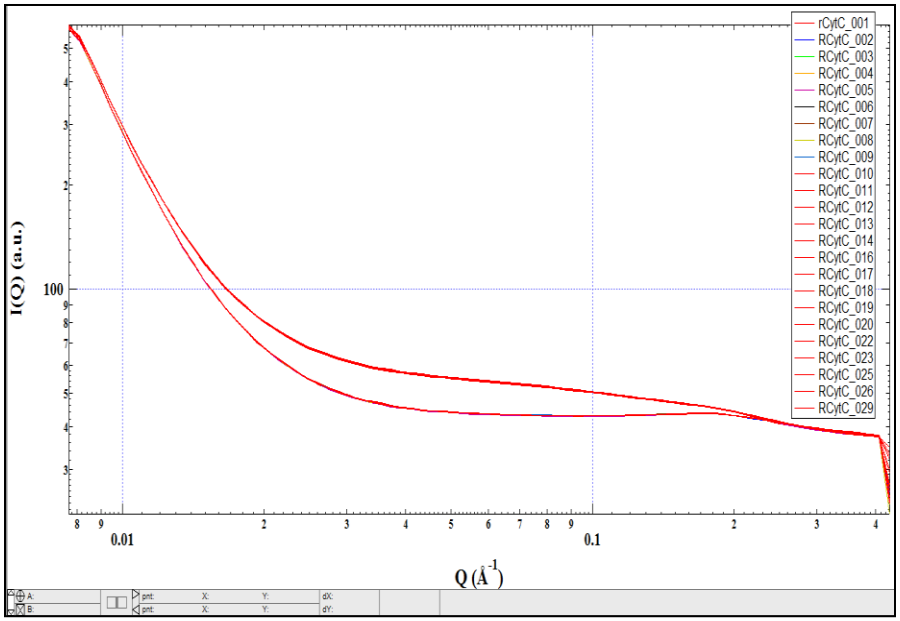
If the shelf life is really short, on-site repurification or LC-SAXS (if available).

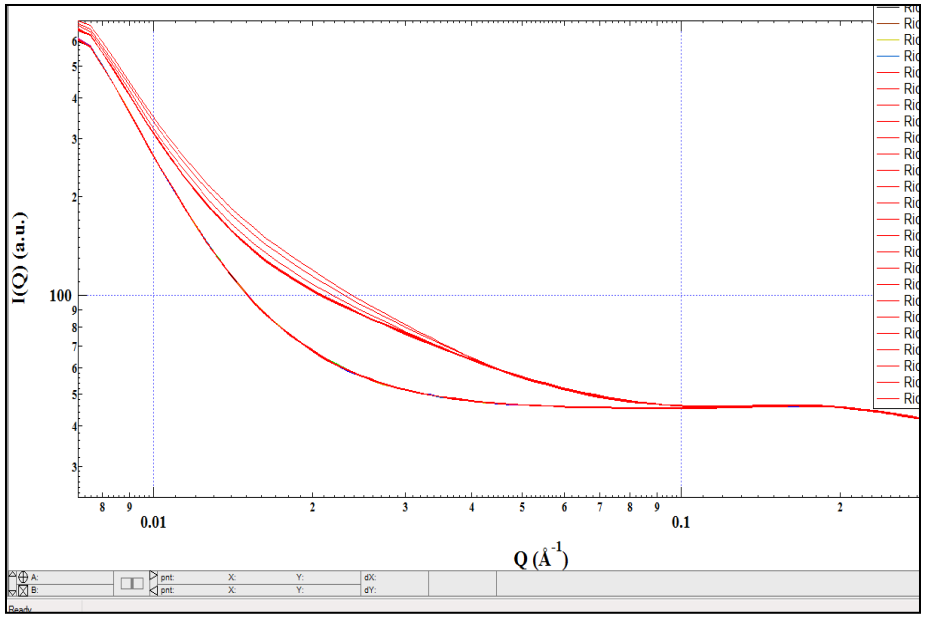
Syringe Filters

Spin Filters

Ultracentrifugation (helps in some cases but may be excessive in most, especially large proteins and complexes)

Radiation Damage

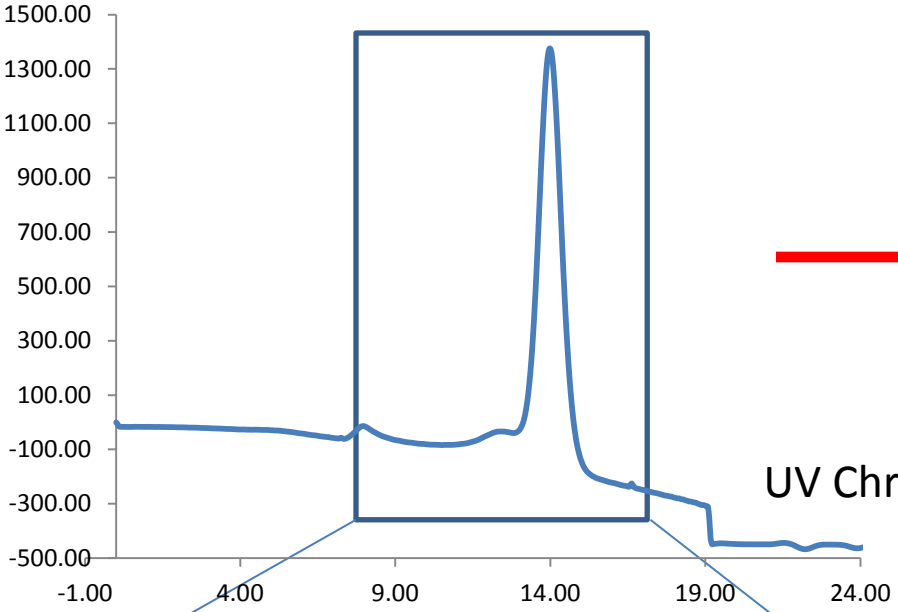




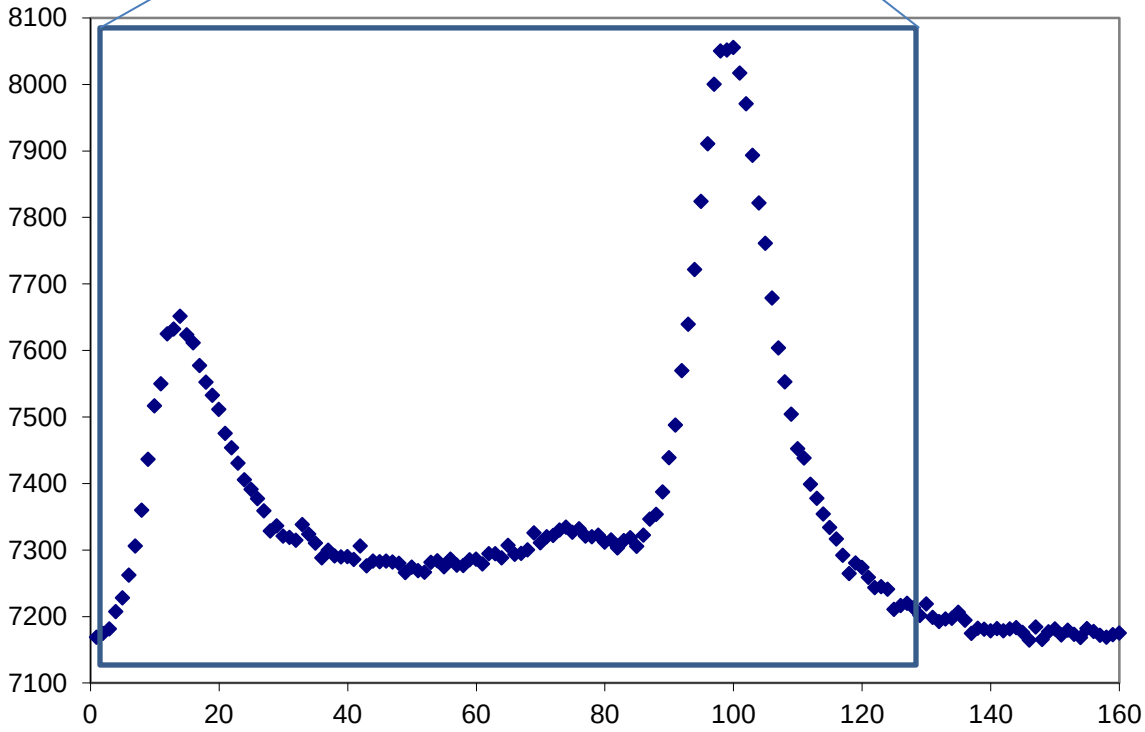
Remedies:

1. Modify buffer composition – a. Add free radical scavengers such as DTT, and TCEP. b. Low percentages (1-5%) of glycerol.
2. Alter Data collection strategy – continuous uni-directional sample flow during exposure (need a lot of sample), reduce exposure time, temperature, and beam attenuation.

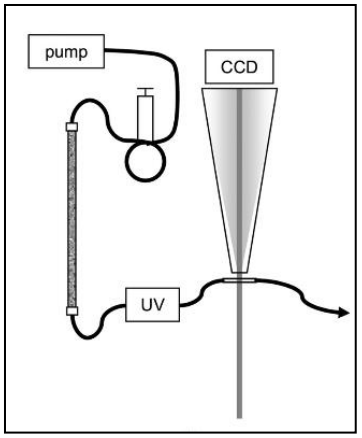
Low-UV signal is not necessarily low scatter.



UV Chromatogram



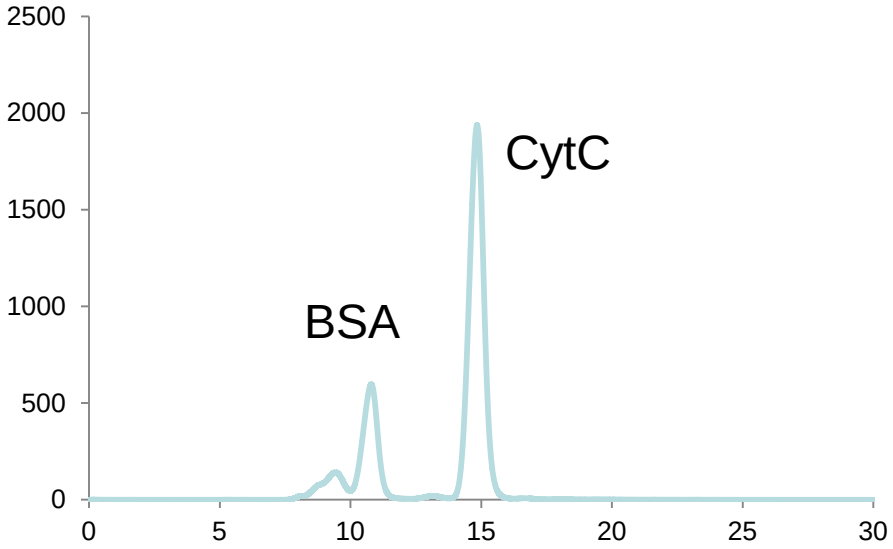
Integrated Intensity of Individual Exposures Plotted in Sequence

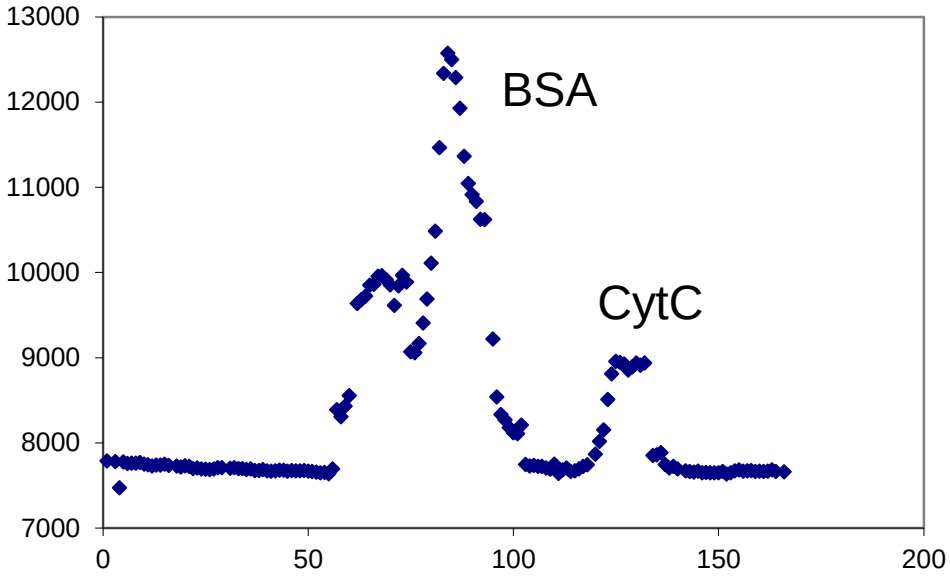


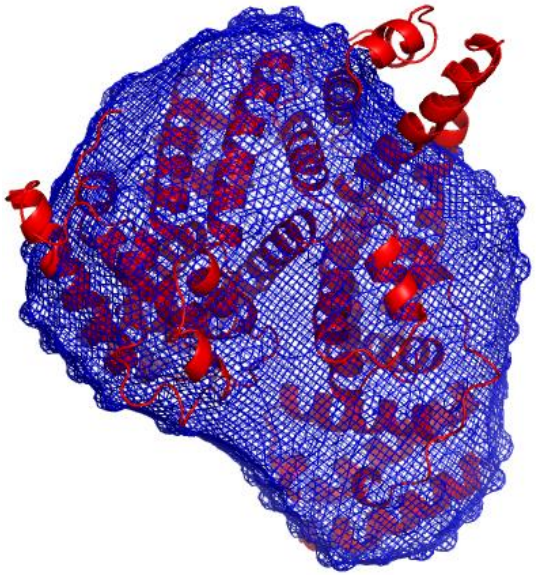
LC-SAXS Configuration Schematic

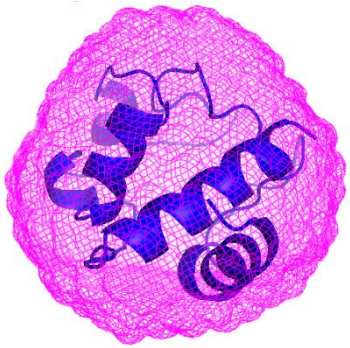
Mathew, E, et al., J Synchrotron Radiat. 2004 Jul 1;11(Pt 4):314-8. Epub 2004 Jun 23.

SEC-SAXS: Truly Efficient Way to Collect Bio-SAXS Data







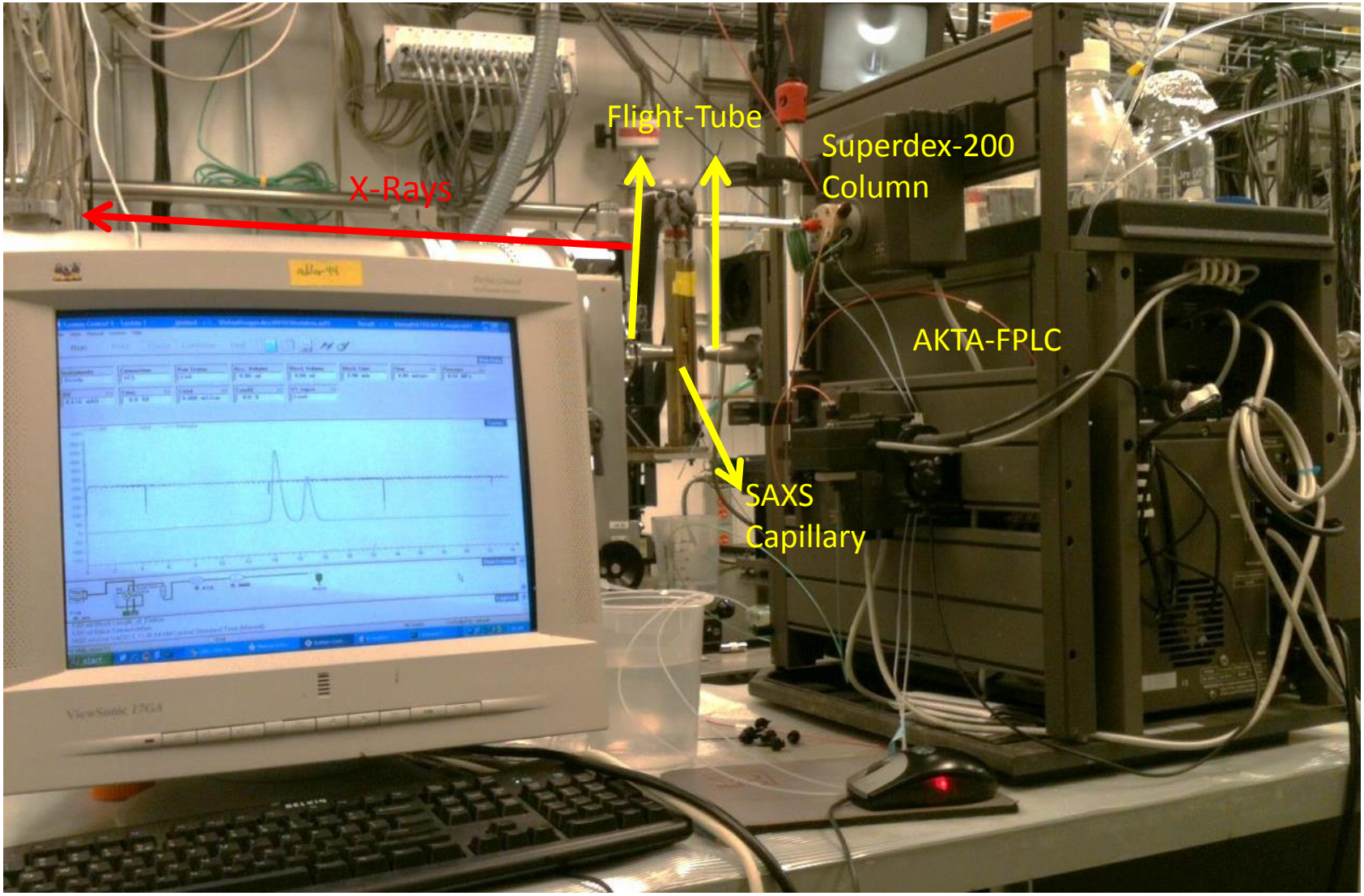


Easy Access to SEC-SAXS Logistics

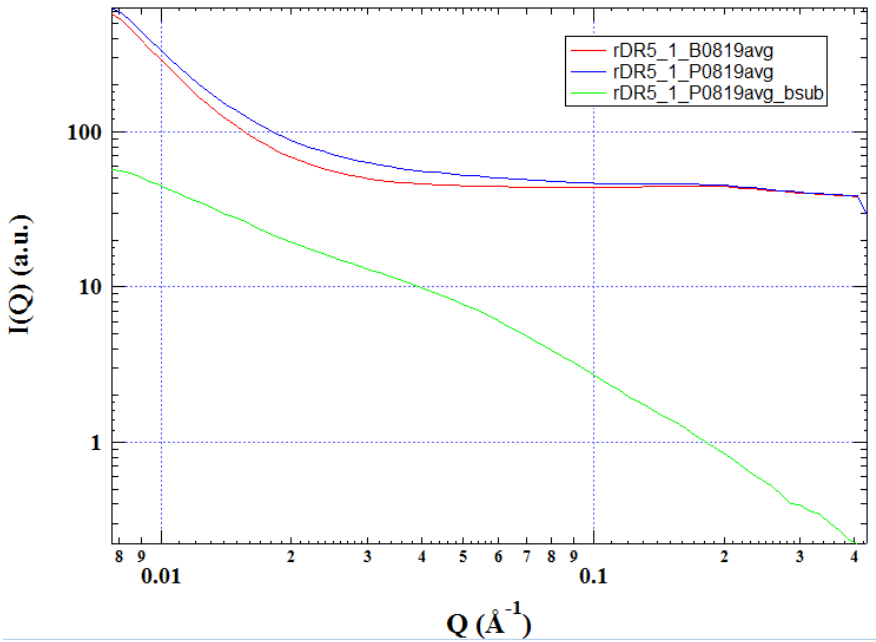
Commercially Available Size-Exclusion Columns Suitable for SEC-SAXS				
Column Name	Exclusion Limit (M_r)	Recommended Flow Rate (ml/min)	Theoretical Plates*	Column Volume (ml)
Superdex-200 Increase 10/300	~ 1.3 X 10^6	0.75	> 48,000 m^{-1}	24
Superdex-200 Increase 5/150	~ 1.3 X 10^6	0.45	> 42,000 m^{-1}	3
Superdex-75 5/150	~ 1 X 10^5	0.15	> 25,000 m^{-1}	3
Superdex-75 10/300	~ 1 X 10^5	0.50	> 25,000 m^{-1}	24
Superose-6 10/300	~ 4 X 10^7	0.50	> 30,000 m^{-1}	24
Superose-6 5/150	~ 4 X 10^7	0.15	> 30,000 m^{-1}	3
Superose-12 10/300	~ 2 X 10^6	0.50	> 40,000 m^{-1}	24
KW 802.5	~ 1.5 X 10^5	1.00	> 21,000 per column	~ 15
KW 803	~ 7 X 10^5	1.00	> 21,000 per column	~ 15
KW 804	~ 1 X 10^6	1.00	> 16,000 per column	~ 15
KW 402.5-4F	~ 1.5 X 10^5	0.33	> 35,000 per column	~ 5
KW 403-4F	~ 6 X 10^5	0.33	> 35,000 per column	~ 5
KW 404-4F	~ 1 X 10^6	0.33	> 25,000 per column	~ 5
KW 405-4F	~ 2 X 10^7	0.33	> 25,000 per column	~ 5

The number of ***theoretical plates*** can be calculated from a chromatographic peak after elution. N = 5.55 tR2/w21/2 .
tR = The time between sample injection and the peak reaching a detector at the end of the column is termed the *retention time.* *w*1/2 is the peak width at half-height.

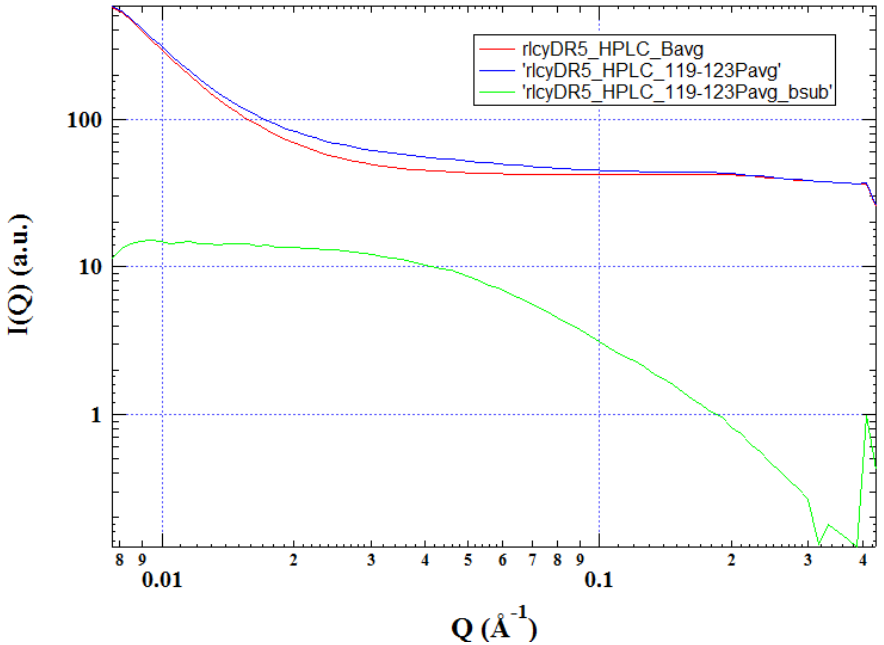
LC-SAXS Setup at BioCAT (APS)

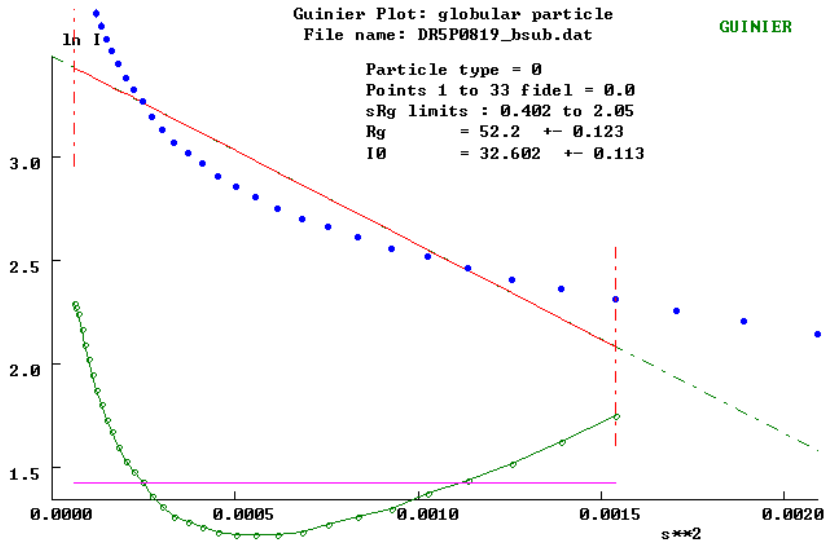


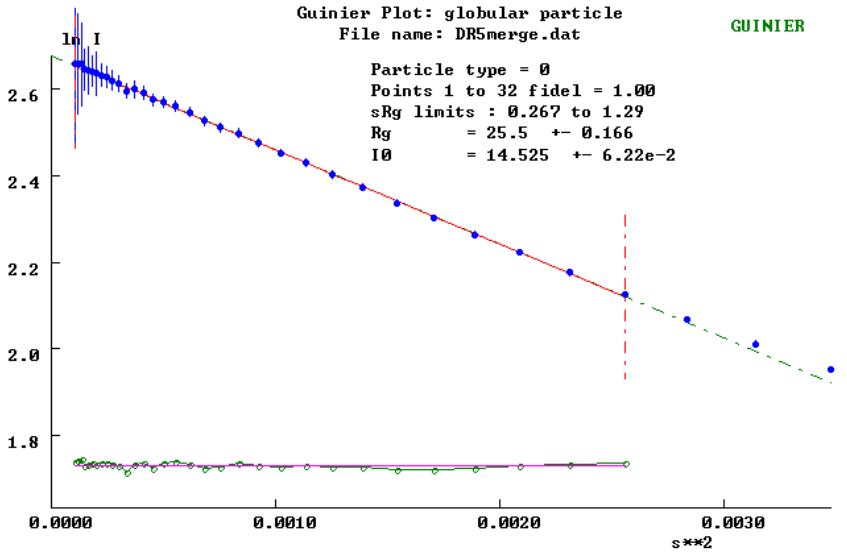
No SEC



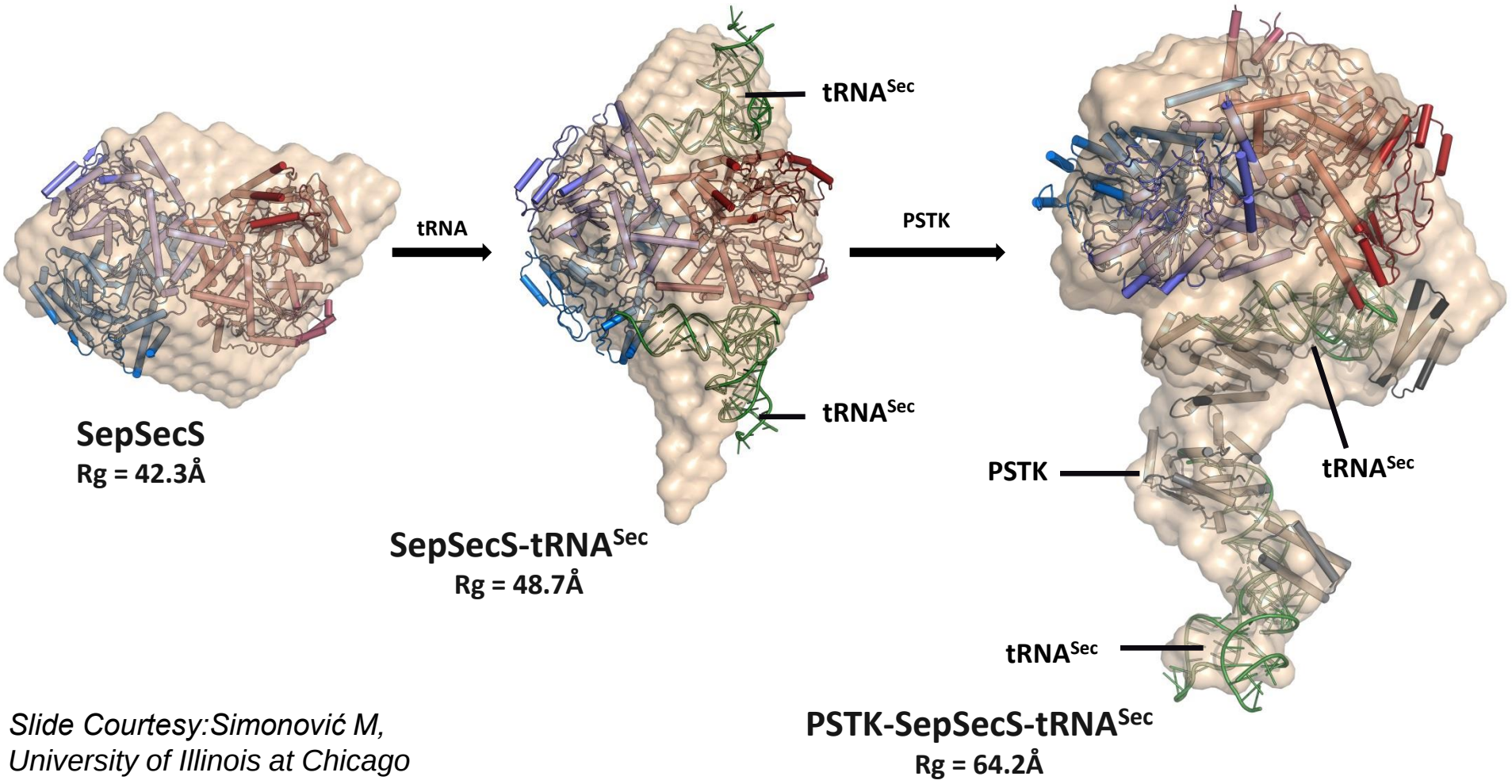
SEC-SAXS







PSTK, SepSecS and tRNA^{Sec} form a stable complex



Slide Courtesy:Simonović M, University of Illinois at Chicago

SEC Instrumentation Upgrades to Facilitate Automation & Combined Techniques

- Acquired an AKTA-pure – modular in design. Therefore amenable to adding elements in the future that will enable automatic sample loading (column valve, loop valve, etc…).

- We will interface with auto-sampler, MALS system from Wyatt Scientific and refractometer etc..

- Already has multiple wavelength UV detector that is capable of measuring absorbance at as many as 3 wavelengths simultaneously.

- Also made available smaller columns which can be used where appropriate to increase efficiency.

LC-SAXS : Advantages

- If right column is chosen, proteins / complexes of interest can be separated from contaminants, degradation products, and/or aggregates immediately before exposure to X-rays.

- The elution peak, if symmetric, and if Rg remains unchanged, represents a dilution series and therefore extrapolation to zero concentration is achieved easily.

- Radiation damage is prevented due to unidirectional flow of sample therefore preventing repeated exposure.

- Buffer blank is obtained quite easily by averaging the regions before and after the peak.